Theor Appl Genet (1999) 98: 1072–1078

X. Shan · T. K. Blake · L. E. Talbert

Conversion of AFLP markers to sequence-specific PCR markers in barley and wheat

Received: 30 June 1998 / Accepted: 26 October 1998

Abstract Conversion of amplified fragment length polymorphisms (AFLPs) to sequence-specific PCR primers would be useful for many genetic-linkage applications. We examined 21 wheat nullitetrasomic stocks and five wheat-barley addition lines using 12 and 14 AFLP primer combinations, respectively. On average, 36.8% of the scored AFLP fragments in the wheat nullitetrasomic stocks and 22.3% in the wheat-barley addition lines could be mapped to specific chromosomes, providing approximately 461 chromosome-specific AFLP markers in the wheat nullitetrasomic stocks and 174 in the wheat-barley addition lines. Ten AFLP fragments specific to barley chromosomes and 16 AFLP fragments specific to wheat 3BS and 4BS chromosome arms were isolated from the polyacrylamide gels, re-amplified, cloned and sequenced. Primer sets were designed from these sequences. Amplification of wheat and barley genomic DNA using the barley derived primers revealed that three primer sets amplified DNA from the expected chromosome, five amplified fragments from all barley chromosomes but not from wheat, one amplified a similar-sized fragment from multiple barley chromosomes and from wheat, and one gave no amplification. Amplification of wheat genomic DNA using the wheat-derived primer sets revealed that three primer sets amplified a fragment from the expected chromosome, 11 primer sets amplified a similar-sized fragment from multiple chromosomes, and two gave no amplification. These experiments indicate that polymorphisms identified by AFLP are often not transferable to more sequence-specific PCR applications.

Communicated by B. S. Gill

X. Shan · T. K. Blake · L. E. Talbert (✉)
Plant Sciences Department, Montana State University, Bozeman, MT 59717, USA
E-mail: usslt@montana.edu

Key words Amplified fragment length polymorphism (AFLP) · Wheat (*Triticum aestivum* L.) · Barley (*Hordeum vulgare* L.)

Introduction

Amplified fragment length polymorphism (AFLP) detection is a DNA fingerprinting technique (Vos et al. 1995) that permits analysis of a subset of restriction fragments from a complete digest of genomic DNA. AFLP analysis entails the digestion of genomic DNA with restriction enzymes, followed by amplification of a subset of the restriction fragments using PCR. PCR products are resolved on denaturing polyacrylamide gels, providing an efficient tool for revealing polymorphisms. The high efficiency, reproducibility and reliability of AFLP has been supported by a number of recent publications. Abundant AFLPs have been found in many plant species, confirming their use in plant genetic studies. AFLP has been used to assess genetic diversity in wheat (*Triticum aestivum* L.) (Barrett and Kidwell 1998; Barrett et al. 1998; Burkhamer et al. 1998), barley (*Hordeum vulgare* L.) (Ellis et al. 1997; Schut et al. 1997), maize (*Zea mays* L.) (Ajmone Marsan et al. 1998), lettuce (*Lactuca* sp. L.) (Hill et al. 1996), sunflower (*Helianthus annuus* L.) (Hongtrakul et al. 1997), pea (*Pisum* sp. L.) (Lu et al. 1996), soybean (*Glycine max* L.) (VanToai et al. 1997), *Manihot* (Roa et al. 1997), and *Eucalyptus urophylle* (Gaiotto et al. 1997). It also has been used to construct high-density genetic maps of barley (*Hordeum vulgare* L.) (Becker et al. 1995; Qi and Lindhout 1997), rice (*Oryza sativa* L.) (Maheswaran et al. 1997), soybean (*G. max* L.) (Keim et al. 1997), and melon (*Cucumis melo* L.) (Wang et al. 1997). AFLP analysis has been used in quantitative trait analysis (Pakniyat et al. 1997; Powell et al. 1997; Roa et al. 1997), as well as in the enrichment of DNA markers near a locus of interest (Ballvora et al.

1995; Meksem et al. 1995; Thomas et al. 1995; Rouppe van der Voort et al. 1997). A comparison of AFLPs with random amplified polymorphic DNA (RAPD) and sequence-tagged microsatellite (SSR) markers (Jones et al. 1997) showed that AFLPs were relatively reproducible.

Despite the reported use of the AFLP technique in various genetic analyses, little information is available regarding the cloning of AFLP fragments for conversion to other marker types. The value of such a conversion is that other types of marker analysis are less expensive and can be more easily employed using large populations. Given that tight linkage between AFLP markers and traits of interest can be identified, it may be useful to convert AFLPs to restriction fragment length polymorphisms (RFLPs) or sequence-specific PCR markers. In the few cases in which AFLP marker conversion has been attempted (Meksem et al. 1995; Cho et al. 1996; Qu et al. 1998), only a few of the corresponding RFLP or sequence-specific PCR markers retained the specificity indicated by the original AFLP markers. Conversion of different types of markers, such as RFLPs, RAPDs and microsatellite markers, to sequence-specific PCR markers has been reported by several authors (Bradshaw et al. 1994; Salentijn et al. 1995; Brady et al. 1996; Talbert et al. 1996; Cheung et al. 1997). However, the efficiency and difficulties associated with conversion of AFLPs are unknown.

In the experiments described in this paper, we attempted to address issues concerning AFLP cloning and the conversion of AFLPs to sequence-specific markers. Several hundred sequence-tagged-site (STS) PCR markers have been developed from different marker types (Talbert et al. 1994; Blake et al. 1996; Erpelding et al. 1996) for use in genetic analysis and marker-assisted selection in wheat and barley. However, regions of chromosomes that are not marked by available primer sets still exist. The conversion of AFLPs to sequence-specific primers would allow further saturation of the wheat and barley genetic maps. The goal of the present study was to determine the feasibility and the efficiency of cloning and converting AFLPs to sequence-specific markers in wheat and barley.

Materials and methods

Plant materials

Twenty one nullitetrasomic stocks (NTs) of 'Chinese Spring' wheat (Sears 1954), five wheat-barley addition lines (WBALs) (Shepherd and Islam 1981), three Chinese Spring wheat ditelosomic stocks (DTs) (Sears 1954), the wheat cultivar Chinese Spring and the barley cultivar Betzes were used for AFLP analysis. WBALs for chromosomes 1, 2, 4, 6 and 7 were used, while WBALs for chromosomes 2 and 5 were not available. Total genomic DNA was extracted from young leaves of greenhouse-grown plants as described by Dellaporta et al. (1983). A single plant was used to represent a genotype.

AFLP analysis

AFLP marker analysis was conducted using AFLP Analysis System I, AFLP Start Primer Kit (Life Technologies, Gaithersburg, Md.), as described by Vos et al. (1995). A total of 250 ng of genomic DNA for each line was completely digested with *Eco*RI/*Mse*I. *Eco*RI and *Mse*I adapters were ligated to the restriction fragments. Primers with one additional nucleotide were used for pre-amplification. Primers with three additional nucleotides were employed for selective amplification. Twelve and fourteen primer combinations were employed for NTs and WBALs, respectively, with *Eco*RI primers labeled with γ-^{33}P-ATP (NEN, Boston, Mass.). Selective amplification products were loaded on 6% polyacrylamide denaturing sequencing gels. Gels were run at 50 W constant power, transferred to Whatman paper, dried, marked with radioactive ink or nicks in film corners for orientation purposes, and exposed to X-ray film (Kodak Biomax-MR) for 16–24 h. Intense bands were scored.

AFLP fragment isolation

Dried gels and films were lined up using radioactive ink marks or nicks to isolate targeted AFLP fragments. A needle was employed to punch around the band of interest through the film to the dried gel. A sharp, clean razor blade was used to excise the selected piece of gel. The DNA-containing gel piece was placed in a 1.5-ml microcentrifuge tube with 100 µl of sterile water for 10 min at room temperature. The water was decanted and replaced with 100 µl of extraction buffer (0.5 M ammonium acetate, 5 mM EDTA). The gel piece was crushed with a pipette tip, boiled for 3 min, and tubes were centrifuged at 14 000 rpm for 5 min. The supernatant was transferred to a new tube and 100 µl of 8 M ammonium acetate, 5 µg of tRNA and 700 µl of cold 100% ethanol were added to precipitate the DNA. Tubes were incubated at -70°C for 30 min then centrifuged for 5 min. The supernatant was removed and pellets washed with cold 80% ethanol. Samples were centrifuged, the supernatant was removed, and pellets were re-suspended in 10 µl of sterile water. One to four microliters were used for PCR.

Cloning of AFLP fragments

Standard PCR protocol was performed in a 50-µl reaction (94°C, 4 min; 30 of 94°C, 1 min, 50°C, 1 min, 72°C, 1.2 min; 72°C, 7 min; 4°C, hold) on each extracted AFLP fragment. Primers were the corresponding unlabeled selective AFLP primers using 0.2 µg per 50-µl reaction. A 10-µl sample of each PCR product was electrophoresed on a 2% agarose gel. The size of each band was compared with that estimated from the AFLP gel. Cloning of PCR products was done using the pCR2.1-TOPO vector (Invitrogen, Carlsbad, Calif.).

Conversion of AFLPs to sequence-specific markers

After cloning, 10–20 white colonies from each transformation were selected and each colony was cultured overnight in 100 µl of LB broth with 50 µg/ml of ampicillin. A 4-µl aliquot of each culture was amplified by PCR as described above using the same set of unlabeled AFLP selective primers. PCR products were digested with 2–4 restriction enzymes and screened on 2%-agarose or 7%-polyacrylamide gels. Selected colonies were sequenced using the Sequenase version 2.0 DNA Sequencing kit (United States Biochemical, Cleveland, Ohio). All sequences contained the *Eco*RI adapter at one end and the *Mse*I adapter at the other end. Based on the sequences of cloned fragments, new primers internal to the AFLP selective primers were designed using the OLIGO program (Rychlik and Rhoads 1989). These primers were employed to amplify

the genomic DNAs of wheat nullitetrasomic stocks with Chinese Spring wheat as a control, or wheat barley addition lines with Chinese Spring wheat and Betzes barley as controls. PCR products were examined on 2% agarose gels or 7% polyacrylamide gels to determine whether the primers amplified a fragment from the same chromosome indicated by the corresponding AFLP markers.

Colony lifting and hybridization

An alternative method for identifying target colonies after cloning for the WBAL experiment was by colony lifting and hybridization. The colonies on the plates were replica-plated, followed by colony lifting and hybridization. Probes were made from total unlabeled AFLP selective-amplification products, with the positive control being the WBAL of the targeted chromosome and the negative control being a WBAL for a non-targeted chromosome. Magnacharge Nylon membrane (Micron Separations Inc.) was used following the manufacturer's instructions. A Prime-It II, Random primer labeling kit (Stratagene, La Jolla, Calif.) was used for probe labeling.

Results and discussion

Identification of chromosome-specific AFLP markers

Wheat chromosome-specific AFLP markers were identified using Chinese Spring nullitetrasomic stocks. For the 12 AFLP primer combinations employed (Table 1), an average of 104 ($\pm$30.9) scorable amplification products per combination was observed. The size range was from 50 to 700 bp. Wheat chromosome-specific AFLP markers were identified as bands missing in only one NT stock but present in all other NTs and in Chinese Spring. A total of 461 wheat chromosome-specific AFLP markers were identified, accounting for 36.8% of the 1253 amplification products scored. The amplified products with no chromosome specificity may either be repetitive or low-copy loci on more than one homoeologous chromosome. The number of AFLP markers assigned to each wheat chromosome were not evenly distributed, ranging from four for 1A to 32 for 3B and 5B (Table 2). The chromosome-specific markers were confirmed by repeating this AFLP analysis. Ditelosomic stocks DT3BS, DT3BL and DT4BS were used to specify the location of AFLP markers to chromosome arms 3BS and 4BS (Fig. 1), since there is a shortage of PCR markers for these chromosome regions (Erpelding et al. 1996). The 3BS- and 4BS-specific AFLP markers were selected for subsequent cloning experiments. The size of AFLP fragments for cloning ranged from 150 to 700 bp.

Table 1 Primer combinations employed

NTs	WBALs
E-A/M-C[a]	E-A/M-C
E-AAG/M-CAC[b]	E-AAG/M-CTA
E-AAG/M-CTA	E-AAG/M-CTC
E-AAG/M-CTC	E-ACG/M-CAC
E-ACC/M-CTA	E-ACG/M-CAG
E-ACC/M-CTG	E-ACG/M-CAT
E-ACG/M-CAC	E-ACG/M-CTA
E-ACG/M-CAG	E-ACG/M-CTC
E-ACG/M-CAT	E-ACG/M-CTG
E-ACG/M-CTC	E-ACT/M-CAC
E-ACT/M-CAC	E-ACT/M-CTC
E-AGC/M-CAG	E-ACT/M-CTG
E-AGC/M-CTG	E-AGC/M-CAG
	E-AGC/M-CTA
	E-AGC/M-CTG

[a] Preamplification primers,
E-A: GACTGCGTACCAATTC-A
M-C: GATGAGTCCTGAGTAA-C
[b] Selective amplification primers,
E-AAG: GACTGCGTACCAATTC-AAG
M-CAC: GATGAGTCCTGAGTAA-CAC

Table 2 Distribution of the numbers of the chromosome-specific AFLPs observed in nullitetrasomic wheat stocks (NTs) and wheat barley addition-line stocks (WBALs)

Wheat stocks	Chromosome-specific AFLPs	Wheat stocks	Chromosome-specific AFLPs
NTs	461	NT-5D	14
NT-1A	4	NT-6A	8
NT-1B	21	NT-6B	22
NT-1D	18	NT-6D	15
NT-2A	30	NT-7A	19
NT-2B	39	NT-7B	22
NT-2D	27	NT-7D	13
NT-3A	30	WBALs	174
NT-3B	32	1	40
NT-3D	23	2	37
NT-4A	20	4	35
NT-4B	34	6	34
NT-4D	18	7	28
NT-5A	20		
NT-5B	32		

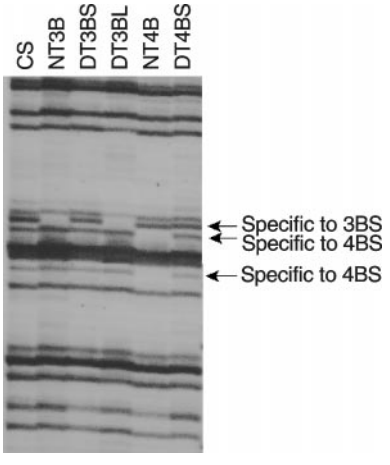

Fig. 1 Mapping chromosome-specific AFLPs to chromosome arms using ditelosomic wheat stocks. The band present in Chinese Spring and DT3BS but absent in NT3B and DT3BL indicates that this band marks the wheat 3BS chromosome arm

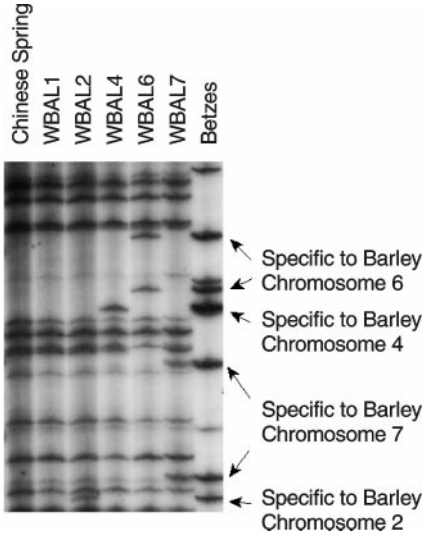

Fig. 2 AFLPs in wheat-barley addition lines (WBALs). The *arrows* indicate barley chromosome-specific AFLPs. A band which is present in Betzes and one WBAL, but absent in Chinese Spring and other WBALs, is considered as a barley chromosome-specific AFLP marker

Five wheat-barley addition lines (WBALs), Betzes barley and Chinese Spring wheat, were used to identify barley chromosome-specific AFLP markers. Fourteen primer combinations were employed for AFLP analysis (Table 1). An average of 56 ($\pm$30.6) scorable barley derived amplification products per combination was observed. A band present in Betzes and one WBAL but absent in Chinese Spring and other WBALs was considered as a barley chromosome-specific AFLP marker (Fig. 2). One hundred and seventy four barley chromosome-specific AFLP markers out of 781 total barley derived bands were scored, with chromosome-specific AFLP markers accounting for 22.3% of the total. The numbers of AFLP markers assigned to each barley chromosome were more evenly distributed than those observed for wheat (Table 2). These barley chromosome-specific AFLP markers were confirmed by repetition of AFLP analysis on the WBALs. Barley chromosome-specific AFLP markers well-separated from surrounding AFLP fragments, ranging in size from 150 to 650 bp, were chosen for subsequent cloning experiments.

Conversion of AFLPs to sequence-specific PCR markers

Results from preliminary experiments showed that inserts in the colonies from a single transformation event were typically not identical. Fifty colonies from each transformation were chosen to perform PCR with the corresponding unlabeled AFLP PCR primers and the amplified inserts were digested with two four-base cutter restriction enzymes. Generally one restriction pattern was shared by a majority of the inserts. Some had two or three restriction patterns occurring at similar frequencies. A few of them had no predominant pattern, with colonies showing high heterogeneity.

An initial set of cloning experiments was with barley chromosome-specific AFLP markers. Ten AFLP fragments selected to be well-separated from surrounding fragments, and marking specific barley chromosomes, were isolated from the AFLP gels, re-amplified, and cloned into the pCR 2.1-TOPO vector. Colonies were screened prior to sequencing, either by restriction analysis or by colony hybridization. Restriction analysis involved selecting 20 colonies from each plate and performing a PCR on each colony, using the original set of unlabeled AFLP selective primers. Amplified products were digested with restriction enzymes and the banding patterns were resolved on 2% agarose gels or 7% polyacrylamide gels. One colony representing the majority banding pattern was selected and sequenced. An alternative strategy to screen the mixed colonies was by colony hybridization. Colonies which hybridized to the positive control (WBAL of the targeted chromosome) but not the negative control (WBAL of a non-targeted chromosome) were selected and sequenced. Two positive colonies from each of ten plates were chosen for sequencing. Fifteen of the twenty colonies selected by colony hybridization were identical to those identified by the restriction-analysis strategy. All ten sequences identified through restriction-fragment analysis were identified by the colony hybridization method. This analysis suggests that the restriction-analysis strategy was as efficient as the more laborious method of colony hybridization. The restriction-analysis strategy has been used successfully to identify target sequences cloned from differential display gels (Zhao et al. 1996).

Primers were designed for the ten sequences identified by both methods (Table 3). Amplification with these sequence-specific PCR primers on the genomic DNA of the wheat-barley addition lines revealed four distinct results. (1) Five primer sets amplified a similar-sized fragment on all barley chromosomes but no fragment in wheat (Fig. 3 A). (2) Three primer sets amplified fragments specific to the predicted barley chromosome (Fig. 3 B). (3) One primer set amplified a similar-sized fragment in both wheat and barley. Polymorphisms existed between wheat and barley upon digestion of these amplified products with *Rsa*I. (4) One primer set gave no amplification.

The second set of AFLP fragments cloned was the wheat chromosome-specific markers. Twelve and four AFLP fragments specific to wheat 3BS and 4BS chromosome arms, respectively, were isolated and cloned. We only used the restriction-analysis method for colony screening on this group because this method proved to be as efficient as the colony hybridization method in screening the mixed colonies in previous experiments. A single colony representing the major

Table 3 PCR primers specific to chromosomes or to genome in wheat and barley

Primer designation	Specificity	Sequence (5′ → 3′)
XD1	Barley	U: AACAAGCTGAGACCCTACTG R: CTACTTGTATCGGTTTATCG
XD2	Barley chromosome 4	U: GTACCAGTATGGCACTCCTC R: GCCCGTAATGCTCATCTTTA
XD3	Barley	U: AGACTTTGATTGGTCATGGC R: TCAAGTGACGAAAAGGAACG
XD4	Barley chromosome 7	U: GGGTTGATGTTTTTGACATG R: AACACATTTGGGGAGAAGAG
XD5	Barley chromosome 4	U: TTGGGAGATGGTGAGGTTAG R: CAAAATCACATGCAACCCAC
XD6	Barley	U: CTCGATGCAACATCATATGC R: CCTAACATATACCTCGTGCA
XD7	Barley (digestion with *Rsa*I)	U: CACACTGCCACAGCATATTA R: ACTCCTCACTGATTCTTCGG
XD8	Barley	U: CATCCCATACATCCAATACA R: AATTCAGCGATATTGTTGGA
XD9	Barley	U: GTTATGCACCTGGAGATGTG R: CGAGCTCCTAGTAGTTGTTG
XJ5	Wheat 3BS	U: GACTCGTGATCGAAATCTTT R: AGAGTGTGAATGCTTCAAGA
XJ26	Wheat 3BS	U: TTGCCTAGTCAATCACTAGT R: TTCTGAATACCAGCATTAGC
XJ28	Wheat 3BS	U: TGTGGAGCAAATCTGCTATT R: AGAGATTCCCGAGATTACAT

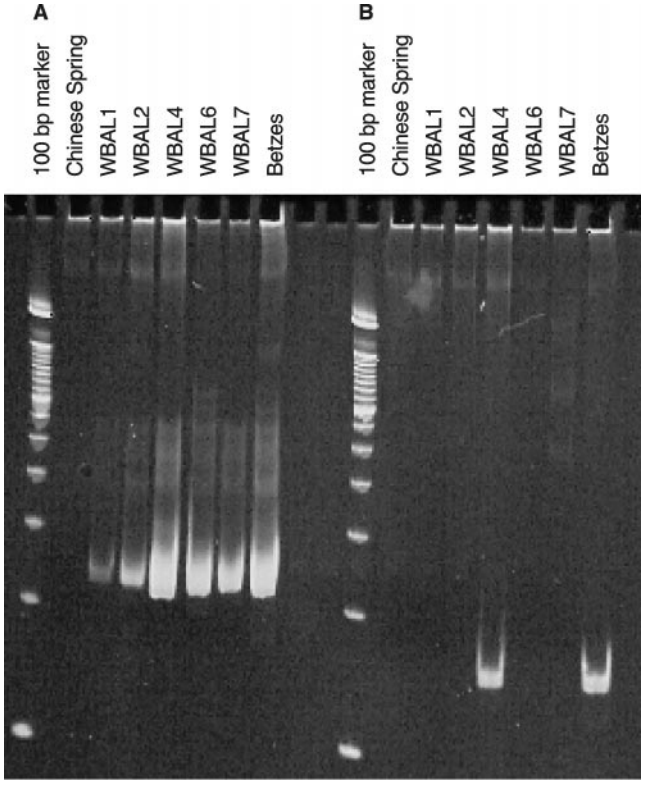

Fig. 3 Panel **A** Primer set XD1was designed from a barley chromosome-4 AFLP marker. XD1 amplifies a similar-sized fragment in all wheat barley addition lines but not in Chinese Spring wheat, indicating that it is specific to barley but not to any particular chromosome. The 100-bp marker contains bands of a size range 100–1500 bp. Panel **B** Primer set XD2 was designed from a barley chromosome-4 AFLP marker. XD2 amplifies a fragment in WBAL4 and Betzes barley but not in any other WBAL or Chinese Spring wheat, indicating that it maps to barley chromosome 4

banding pattern from each cloning reaction was chosen for sequencing.

Primers were designed from the sequence data (Table 3) and used for PCR on wheat genomic DNAs. Out of 12 AFLP wheat 3BS markers cloned, three primer sets amplified fragments specific to the wheat 3BS chromosome arm. Two of these also amplified an additional fragment that was not chromosome-specific. One primer set amplified a single, chromosome-specific band (Fig. 4). Eight primer sets amplified fragments with no specificity. One primer set gave no amplification. Of the four AFLP 4BS markers cloned, three primer sets amplified fragments on all wheat NT stocks tested, whereas one primer set gave no amplification.

While chromosome-specific primer sets were obtained through these studies, it is not clear that a single locus was amplified. To test this, we searched for polymorphisms in two wheat mapping populations and one barley mapping population for the wheat and barley derived primer sets, respectively. The amplified products were not polymorphic in the populations for most of the primer sets, even after digestion with a battery of restriction enzymes. The exception to this was primer set XJ28, where the amplified products revealed polymorphisms among the parental genotypes of the Opata 85-Altar 84 mapping population (Nelson et al. 1995). However, the observed segregation for parental types among recombinant inbred lines derived from the cross was 25:12. This does not fit the expected 1:1 ratio, and we are unable to conclude that XJ28 amplifies a single locus.

The cloning of AFLPs has been described in only a few reports. Meksem et al. (1995) reported the cloning of two AFLP markers co-segregating with the *R1* locus

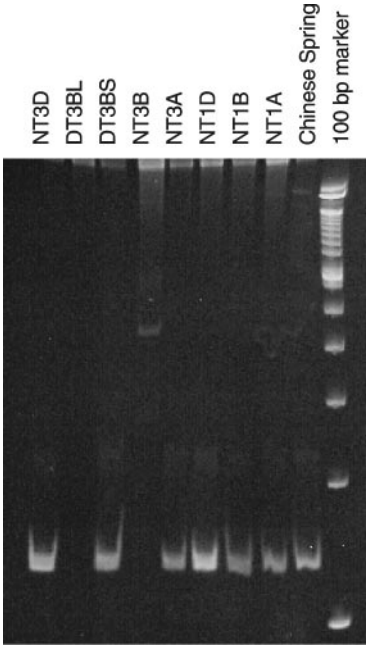

Fig. 4 Primer set XJ28 was designed from a wheat ditelosomic 3BS AFLP marker. XJ28 amplifies a single-copy fragment in all nullitetrasomic stocks (only nullitetrasomic chromsomes 1 and 3 are shown) except NT 3B. Additionally, it amplifies the same fragment in ditelosomic 3BS but not 3BL. This indicates that XJ28 maps to wheat chromosome arm 3BS

on chromosome V of potato and their conversion to STS-PCR markers and RFLP probes. Neither of the STS-PCR markers allowed the identification of alternative alleles. One RFLP probe revealed repetitive fragment patterns on a genomic Southern blot. The other RFLP probe detected a single-copy sequence in potato, co-segregating with the *R* allele. Because the AFLP markers they cloned were 120 bp and 80 bp in length, respectively, they postulated that the STS-PCR products were too small to detect polymorphisms. Qu et al. (1998) described the cloning of six AFLP fragments corresponding to the Chinese Spring *ph1b* deletion and their conversion to PCR-based markers. One primer generated a product from Chinese Spring but not from the *ph1b* deletion line.

Our experiments showed that after cloning 26 wheat or barley chromosome-specific AFLP markers and converting them to sequence-specific primers, six primer sets retained the specificity indicated by the AFLP markers. A possible reason for the lack of efficient conversion may lie with the nature of the AFLP polymorphisms. Primers were generated from sequences internal to the AFLP primers. Nucleotide differences specific to the AFLP primers may have resulted in the chromosome specificity of AFLP bands. This specificity would be lost when internal primers are derived. Additionally, AFLP polymorphisms related to *Eco*RI or *Mse*I restriction-site differences will not be reflected in primers developed from an internal sequence. Thus, while feasible, development of sequence-specific primers from AFLP markers is not an efficient process in wheat.

Acknowledgements This work was partially supported by National Science Foundation Grant #OSR-9350546 and by the Montana Agricultural Experiment Station. We thank Steve Larson for advice and assistance.

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